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AD._____

Award Number: DAMD17-99-1-9111

TITLE: AP-1 Activity in Tamoxifen-Resistant Human Breast Tumors

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REPORT DATE: September 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Annual Summary (1 Sep 99 - 31 Aug 00)

4. TITLE AND SUBTITLE
AP-1 Activity in Tamoxifen-Resistant Human Breast Tumors

5. FUNDING NUMBERS
DAMD17-99-1-9111

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
The hormonal therapy of choice for the treatment of breast cancer has been the antiestrogen tamoxifen. However, tumors eventually acquire a tamoxifen-resistant or tamoxifen-stimulated phenotype, resulting in disease recurrence. Estrogen receptor (ER) is known to interact with other transcription factors such as AP-1 through protein-protein interactions. AP-1 responds to a variety of extracellular signals, including oxidative stress and growth factor stimulation and regulates a variety of genes that could be associated with cellular growth resistant deregulation and transformation. Tamoxifen has been shown to induce oxidative stress and tamoxifen resistant ER positive cell lines are associated with increased AP-1 binding, suggesting that enhanced AP-1 activity can account for tamoxifen-stimulated growth. In this study, I focus on the role of oxidant stress-mediated interactions between ER and AP-1. I have begun development of a novel in vitro transcription and translation assay. I have also begun studying the possible induction of a stress-induced proteasome-ubiquitin ER degradation pathway that may be detectable in human breast tumor samples. Finally, I have completed an initial analysis of 71 breast tumor extracts for key molecular parameters of oxidative stress and growth factor signaling including AP-1 DNA binding, Sp1 DNA binding and protein content, and Erk5 activation.

14. SUBJECT TERMS
Breast Cancer, tamoxifen, estrogen receptor, AP-1, Sp1, BMK(Erk5)

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

20010302 058

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INTRODUCTION

The hormonal therapy of choice for the treatment of breast cancer has been the antiestrogen, tamoxifen. However, tumors eventually acquire a tamoxifen-resistant or tamoxifen-stimulated phenotype, resulting in disease recurrence. Studies of the estrogen receptor (ER) have shown that in many cases expression of a fully functional wild-type receptor continues at relapse and there is experimental evidence that tamoxifen's partial agonist activity may be responsible for stimulation of tumor re-growth. (1). As well, for nearly a third of primary ER positive breast tumors the extracted ER is unable to bind to its cognate DNA estrogen response element (ERE), an effect that we have found may be due to preferential oxidation of zinc finger 2 in the ER DNA binding domain (2), but which may not prevent ligand activated ER gene regulation via tethering to another DNA bound transcription factor complex such as AP-1. ER is known to interact with the activator protein-1 (AP-1) transcription factor complex through protein-protein interactions independent of ERE mediated ER DNA binding, and in certain ER positive cells this may allow tamoxifen to exert an agonist response on AP-1 regulated genes. AP-1 responds to a variety of extracellular signals, including oxidative stress and growth factor stimulation. Genes that are known to be transcriptionally upregulated by AP-1 include collagenase, cathepsin D, and the p-glycoprotein multidrug resistance gene; therefore, enhanced AP-1 activity may be associated with cellular growth resistant deregulation and transformation to a more malignant and invasive phenotype. Tamoxifen has been shown to induce oxidative stress (3), and tamoxifen ER positive cell lines are associated with increased AP-1 binding, suggesting that enhanced AP-1 transcriptional activity can bypass hormone-dependence in ER positive tumors and account for tamoxifen-stimulated growth. Recent analysis of data has shown that ER expression and ER DNA binding are preserved in tamoxifen resistant breast tumors (4). AP-1 DNA binding and the activity of the AP-1 activator Jun N-terminal kinase (JNK) are also increased. In this study, I focus on the role of oxidant stress-mediated interactions between ER and AP-1 in determining tamoxifen resistance in ER positive breast cancers. To date, I have begun development of a novel in vitro transcription and translation assay as proposed. I have also begun studying the possible induction of a stress-induced proteasome-ubiquitin ER degradation pathway that may be detectable in human breast tumor samples, providing a possible mechanism for earlier observations of ER (meroreceptor) ligand binding in tumor samples without detectable DNA-binding, full-length (67 kDa) ER protein. Finally, I have completed an initial analysis of 71 cryopreserved primary breast tumor extracts for key molecular parameters of oxidative stress and growth factor signaling including AP-1 DNA binding, Sp1 DNA binding and protein content, and Erk5 activation.

Progress Overview:

Progress during year 01 as proposed in the original statement of work as well as unexpected findings are discussed below.

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Technical Objective 1: Develop a new assay to measure AP-1 transcriptional activity as a correlate of increased AP-1 DNA-binding and JNK activity in human tumor samples.

The new assay is based on a two step process: The first step is to use a luciferase reporter construct in vitro transcription. The second step is to measure luciferase activity after in vitro translation. As proposed, a (AP-1)4-TK-CA plasmid (and one mutated to inactivate the tandem AP-1 response elements) obtained from H. Rochefort was modified into an (AP-1)-TK-LUC plasmid. The luciferase transcripts were then be purified (proteinase-K digestion, phenol/chloroform extraction followed by ethanol precipitation) and used to prime a rabbit reticulocyte in vitro translation system (Promega) that generates the luciferase product. The amount of product was quantified by luminometer in a standard luciferase assay.

To develop the assay, AP-1 containing nuclear and whole cell extracts from MCF-7 cell lines were used. The levels of AP-1 activity were compared in control vs. 12-O-tetradecanoylphorbol-13-acetate (TPA)-pretreated MCF-7 cells. TPA is a tumor promoter which induces expression of Jun and Fos family members, and enhances both AP-1 DNA binding and transcriptional activity (1); therefore the cells pretreated with TPA should have higher activity level. Transient transfections using the WT and MUT 4AP1/TK/Luc plasmids showed that the plasmids were able to induce Ap-1 activity in whole cell extracts from uninduced and TPA-induced MCF7 cells. TPA caused a 23-fold increase in Ap-1 activity from the WT plasmid (4527 ± 3910 vs 195 ± 63 relative luminosity units) but did not increase activity and in fact appeared to inhibit activity from the MUT plasmid (181 ± 113 vs 35 ± 29). Based on this data, we attempted to measure luciferase activity after the in vitro transcription/translation protocol. Using the 4AP1/TK/Luc constructs, no difference in luciferase activity was detectable in TPA-treated MCF7 cell extracts (Mutant: 9.1 ± 14.8 vs Wildtype: 5.3 ± 8.8). Therefore, although all of the components of the transcription/translation pathway are present in the MCF7 cells as demonstrated by the transient transfection experiments, we were unable to measure an increase in AP-1 activity by TPA in the in vitro assay.

Because the TK promoter has binding sites for other transcription factors that could be masking the effects of TPA, we made a new plasmid construct by replacing the TK promoter for the viral major late promoter E1b that does not contain additional transcription factor binding sites. Using this construct for transient transfection of MCF7 cells treated with and without TPA, we found that the E1b-containing promoter did have lower baseline luciferase activity compared to the TK-containing promoter. Furthermore, similar to the TK construct, TPA induced a major increase in Ap-1 activity from the wildtype, but not mutant, construct. Unfortunately, no difference in Ap-1 activity was seen between TPA-stimulated WT and MUT 4AP1/E1b/Luc (Mutant: 29.0 ± 12.9 vs WT: 28.0 ± 9.1).

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Summary

The in vitro transcription assay remains challenging and is still under development. While increased luciferase activity with TPA stimulation of both the 4AP1/TK/Luc and 4AP1/E1b/Luc plasmids is measurable in transient transfections, no increase was detected in the in vitro assay. Chromatinization has now been shown to be required for proper transcription and translation in several in vitro systems using a variety of promoters (ref). In particular chromatin structure is required to set low-level baseline promoter activity. One reason our in vitro assay is not performing as expected is that despite the decrease in baseline activity of the E1b vs TK construct, chromatinization is probably required. One unsuccessful attempt to form chromatin structures from the 4AP1/E1b/Luc construct has been made in the Tjian laboratory at UC Berkeley, where the process was developed. The protocols for chromatinization have been published but require 6-12 months to develop optimally performing reagents. Thus, we will continue to monitor progress in and consult with the Tjian lab who have been collaborating in our assay development effort. Through this joint effort and before the completion of this project period we hope to establish the extent to which plasmid chromatization will result in successful development of our proposed in vitro transcription assay.

Technical Objective 2: Compare ER DNA-binding with ER isoform content, AP-1 DNA-binding and transactivation, and associated JNK activity from primary tumor samples.

The primary tumor sample extracts analyzed for this portion of the study were obtained through collaboration with Dr. Urs Eppenberger (Basel, Switzerland). This first group of samples (n=71) were all untreated breast tumors selected for ER content ranging from 20-100 fmol/mg.

1. Loss of ER DNA-binding by a unique ER degradation pathway

DNA binding by full-length (67 kDa) ER was not measurable in many of the cryopreserved samples, presumably due to proteolytic degradation of full-length ER leaving only the truncated ligand-binding and c-terminal immunoreactive meroreceptor form of ER. The tumor extracts were intentially prepared without protease inhibitors and were cryopreserved at –70C, suggesting ER degradation as the reason for the lack of ER DNA binding by full-length ER. Two recent papers reported that redox-sensitive ER degradation can occur via a specific proteasome-ubiquitin pathway (5,6). To determine if the ER proteasome-ubiquitin degradation pathway was activated in these tumor extracts, we incubated recombinant ER (Panvera) in selected tumor extracts or homogenization buffer (as control) and then looked for changes in detectable 67 kDa ER by Western analysis. Using this assay, we saw more relative degradation of 67 kDa ER after incubation in the tumor extracts compared to buffer. We next added various classes of protease inhibitors (antipain, leupeptin, PMSF, phenylchloromercuric benzoic acid, phenylchloromercuric sulfonate, chymostatin, EDTA, EGTA) and proteasome inhibitors

(MG132, lactocystin). Preliminary results suggested that only the proteasome inhibitors prevented the degradation of 67 kDa ER in this assay. Future efforts will continue to develop this assay to quantify the activity of this unique redox-sensitive ER degradation pathway in these tumor extracts and to correlate this activity with other redox parameters being measured in these samples (see below).

2. AP-1 DNA binding

AP-1 was measured from these same tumor samples. As an internal positive control, we began measuring DNA binding of the ubiquitous transcription factor, Sp1 and found that the binding of Sp1 varied from sample to sample. Sp1 is known to be an age-related, redox-sensitive transcription factor whose DNA binding zinc fingers are also likely susceptible to thiol specific oxidation. We pursued this unexpected observation by measuring Sp1 DNA binding by EMSA and Sp1 protein by western analysis as well as transcription factor NFK-b DNA binding and the protein kinase Erk5 content, two other oxidant-stress sensitive factors to look for correlation with each other and with patient age at tumor diagnosis (see below). An abstract describing this data was presented at AACR 2000 and a manuscript is in preparation (see Appendix).

3. Sp1 DNA binding, Sp1 protein content, NFK-b DNA binding.

The amount of Sp1 DNA binding varied from sample to sample. Two Sp1-specific bands were detected as determined by oligonucleotide competition and Sp1 supershifting experiments. A third band comprised mostly of Sp3 was identified using a Sp3-specific antibody. In western analysis, two bands were detected of approximately 95 and 106 kDa. These two bands have been identified as differentially phosphorylated forms of Sp1 (7). The amount of each form as well as the relative amounts varied from tumor to tumor.

3. Big Map Kinase (BMK/Erk5)

Western analysis of Erk5 identified two bands of approximately 120 and 125 kDa which have been previously identified as nonphosphorylated and phosphorylated Erk5, respectively (8). The amounts of these two forms varied from sample to sample.

Summary

Although the primary breast tumor samples were selected for moderate ER content, full length (67 kDa) ER was not detectable by western analysis and no DNA binding was detected by EMSA; however, c-terminal immunoreactivity and ER ligand-binding were detectable, suggesting that specific ER degradation had occurred. During the Sp1 Western experiments, a tertiary system was used: primary monoclonal antibody followed by biotinylated anti-mouse antibody followed by horseradish peroxidase (HRP) labeled streptavidin. Using this method, the sensitivity was increased at least 100-fold. We

estimate that only tens of nanograms of ER can be loaded per lane. Control experiments show that the standard primary antibody followed by HRP-labeled secondary western protocol enabled detection of >.50 ng recombinant ER. Therefore, the tertiary system might enable more sensitive detection of ER in the samples, but a more interesting observation worth pursuing is the likely activation of an ER specific ubiquitin-proteasomal degradation pathway. Thus, further experiments are planned to quantitate and verify the initial observations about the various x and y protease inhibitors used and that only the proteasome inhibitors arrested ER degradation in our assay. Optimization of this new ER degradation assay will become a new priority of this overall study and if proven clinically relevant will be given priority over the initially proposed in vitro transcription assay.

Thus, a preliminary summary of the statistical analysis correlating breast tumor (n=71) Sp1 DNA binding, protein content, AP-1 DNA binding, and Erk5 protein content with patient age and other known tumor parameters is as follows:

1. AP-1 DNA binding did not correlate with age nor any of the measured parameters. Increased AP-1 DNA binding may be less dependent on age or oxidative stress than on increased tumor EGFR and/or ErbB2 expression (EGFR and ErbB2 data from Basel group).
2. There was no correlation between NFK-b DNA binding and any of the measured parameters.
3. Increased phosphorylated Erk5 is found in older patients and is associated with loss of Sp1 DNA binding.

Conclusion

As proposed, I have begun development of a novel transcription translation assay. To date, two different 4AP1/Luc constructs have been made and tested for the early feasibility assessment of a potential in vitro transcription assay. Collaborations with the UCB Tjian lab were put into place for assistance in reagent production and assay development. Further development will continue into year 02. Analysis of ER content, ER DNA binding, AP-1 DNA binding, Sp1 DNA binding, Sp1 protein content, and Erk5 activation in a preliminary set of 71 outcome-linked and otherwise characterized tumor extracts has been completed, a summary of the data can be found above. An unanticipated finding, that of a potentially oxidative-stress induced ER-proteasome pathway was discovered. Reagent development and prototype testing of a new ER degradation assay has been initiated which may provide a mechanistic link between oxidant stress-induced loss of ER DNA binding and subsequent ER degradation.

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APPENDIX

Key Research Accomplishments:

1. Two different 4AP1/Luc constructs have been made and tested for the early feasibility assessment of a potential in vitro transcription assay; collaborations with the UCB Tjian lab were put into place for assistance in reagent production and assay development.
2. Analysis of ER content, ER DNA binding, AP-1 DNA binding, Sp1 DNA binding, Sp1 protein content, and Erk5 activation in a preliminary set of 71 outcome-linked and otherwise characterized tumor extracts has been completed.
3. The discovery, reagent development, and prototype testing of a potentially new ER degradation assay has been initiated which may provide a mechanistic link between oxidant stress-induced loss of ER DNA binding and subsequent ER degradation.

Reportable Outcomes:

1. Quong JN, Eppenberger-Castori S, Birrer M, Eppenberger U, Benz CC. Redox-sensitive kinase (Erk5) activation and altered transcription factors (Ap-1, SP1) DNA binding in untreated primary breast tumors. American Association of Cancer Research 2000 Meeting, April 1-5, San Francisco, CA.
2. Eppenberger-Castori S, Moore D, Quong J, Thor A, Eppenberger U, Benz CC. Age-dependent breast cancer prognostic markers. (accepted for presentation, 23rd Annual San Antonio Breast Cancer Symposium (Dec. 2000).
3. Manuscript (in preparation).

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